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This research is centered on testing the hypothesis that there are novel mechanisms in human breast cancer involving functional inactivation of wild-type p53 besides such direct genetic alteration. The immediate goal of this research is to characterize cellular activities which affect the ability of p53 to bind to DNA in a sequence-specific manner. The long term goal is to determine the relevance of such regulators in human breast cancer. Human breast tumor samples would be screened for alterations in the expression of these proteins which affect the DNA binding activity of p53. The identification of proteins which regulate wild-type p53 is an important focus for breast cancer research since the regulation, mechanism of action, and metabolism of such proteins would be central to our understanding of breast cancer and the aberrant expression of such proteins would represent novel important mechanisms of carcinogenesis.

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James L. Manfredi 8/28/99

PI - Signature Date

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Publication resulting from this research:

Thornborrow, E.C. and Manfredi, J.J. (1999) One mechanism for cell-type specific regulation of the *bax* promoter by the tumor suppressor p53 is dictated by the p53 response element. J. Biol. Chem. **274**: 33747-33755.

Meeting abstracts resulting from this research:

Resnick-Silverman, L., St. Clair, S., Thornborrow, E., Maurer, M., Meng, J., Ream, A., and Manfredi, J.J. (1999) Target gene selection by p53 is regulated by multiple mechanisms. ICGEB Workshop: "p53: Twenty Years On" (Trieste, Italy).

ANNUAL REPORT - YEAR 2
Regulation of the tumor suppressor activity of p53 in human breast cancer
IDEA
(08/01/98-07/31/99)

INTRODUCTION

Genetic alteration of p53 resulting in loss-of-function is a common event in human cancer. This research is centered on testing the hypothesis that there are novel mechanisms in human breast cancer involving functional inactivation of wild-type p53 besides such direct genetic alteration. Consistent with its function as a transcription factor, the ability to bind to DNA has been shown to be central to the tumor suppressor activity of p53. Thus, the immediate goal of this study is to identify and characterize activities in human cells which affect the ability of p53 to bind to DNA in a sequencespecific manner. During Year 1, the high mobility group protein HMG-1 was shown to stimulate the ability of p53 to bind to its sequence-specific binding site and two novel factors were identified which bind to a subset of p53 response elements in a sequencespecific manner. Studies in Year 2 have focussed on these latter factors. Electrophoretic mobility shift assays suggest that the binding of p53 and these latter nuclear factors may be mutually exclusive. This suggests that the interaction of these factors with a subset of p53 response elements may regulate p53 target gene selectivity. To explore this notion, in Year 3, these factors will be purified and cloned and their role in p53dependent gene expression will be examined.

BODY

Results

Two novel factors which bind to a subset of p53 response elements in a sequence-specific manner have been identified. Nuclear extract from the p53-null MDAMB-453 cells was used in an electrophoretic mobility shift assay with the *bax* site as radiolabeled probe. Four distinct nuclear factors bound this oligonucleotide (Figure 8A). Competition studies were performed using unlabeled full-length *bax* site as well as the individual half-sites derived from the *bax* element (Oligos A, B, and C) and the *p21* 5′ site. Two of these factors, labeled ns#1 and ns#2, did not display sequence-specificity wher eas the binding of bob1 and bob2 (Binder Of Bax) were effectively competed only by the full-length *bax* site and Oligo B (Figure 1A). Utilizing nuclear extract derived from Hela cells, further competition studies demonstrated that bob1 and Bob2 bound well to the *bax* and mdm-5′ sites, less well to the *p21* 3′ site, the promoter site from the cyclin G gene, and the site located in the second intron of the IGFBP3 gene, but bound poorly, if at all, to the other sites tested (Figure 1B). These results were confirmed using each of these sites as radiolabeled probe (data not shown). Preliminary evidence suggests that these factors appear to be distinct from p53 and its homologs. First, the factors were identified in three different p53-null cell lines and the band-shifts were unaffected by the presence of anti-p53 antibodies (data not shown). Second, bob1 and bob2 bind with high affinity to the *bax* site, which has been previously characterized as being a relatively weak p53 binding site (Figure 1). Third, these factors fail to bind to the *p21* 5′ site which has been shown to mediate transcriptional

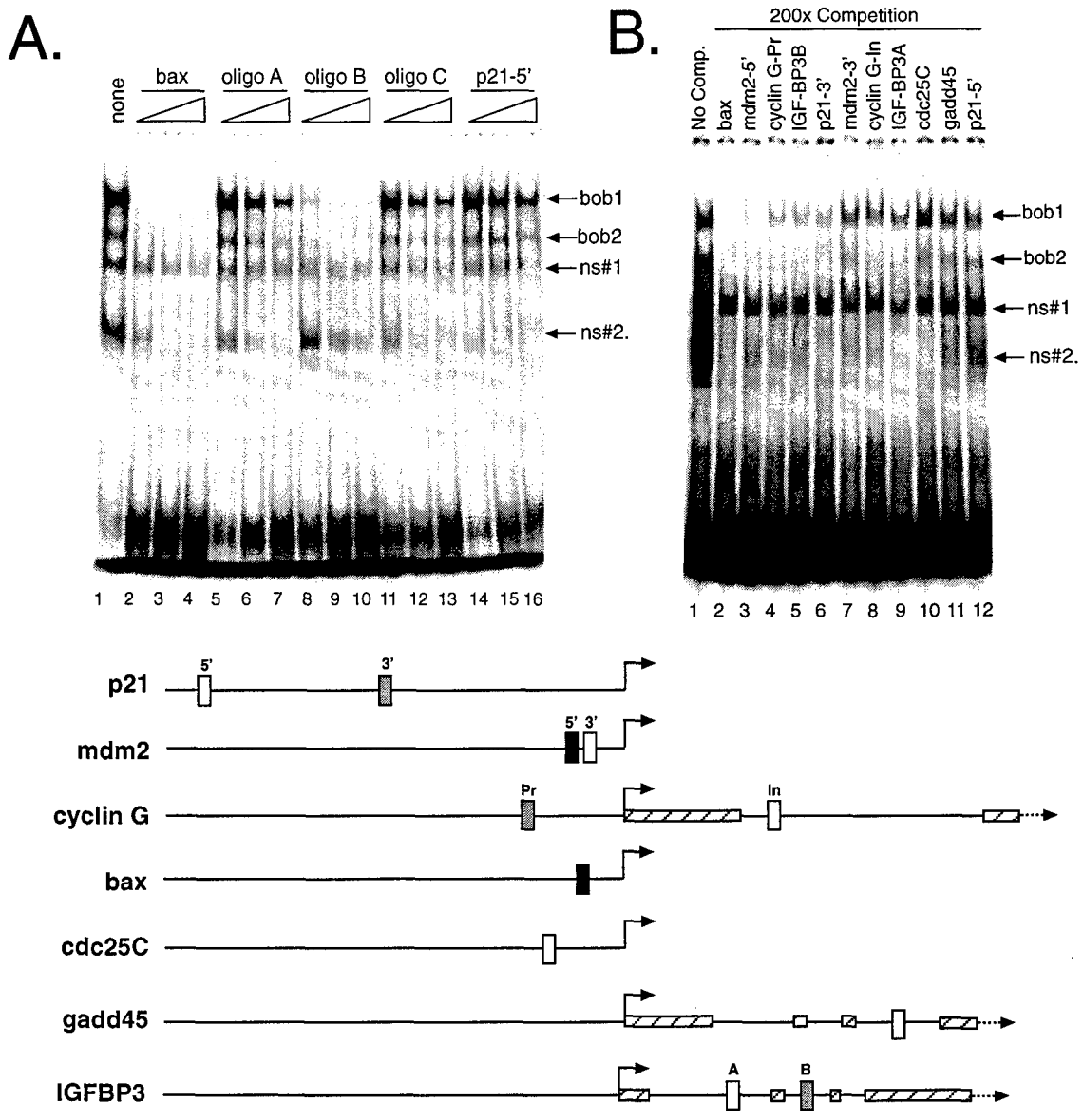

Figure 1. Nuclear extracts contain two factors which bind in a sequence-specific manner to a subset of p53 response elements.

(A) An electrophoretic mobility shift assay was performed using the bax oligonucleotide as radiolabeled probe. Nuclear extract from MDA-MB-453 cells was incubated with probe alone (lane 1) or in the presence of a 10- (lanes 2, 5, 8, 11, 14), 50- (lanes 3, 6, 9, 12, 15), or 100-fold (lanes 4, 7, 10, 13, 16) molar excess of the indicated unlabeled competitors. Oligo A, B, and C, represent shorter sequences derived from the bax element.

(B) An electrophoretic mobility shift assay was performed using the bax oligonucleotide as radiolabeled probe. Nuclear extract from Hela cells was incubated with probe alone (lane 1) or in the presence of a 200-fold (lanes 2-12) molar excess of the indicated unlabeled competitors.

bob1 and bob2 indicate the positions of the two sequence-specific DNA-binding factors, and ns#1 and ns#2 indicate the position of two nonspecific bands. Below the autoradiograms is a schematic drawing of various genes indicating the positions of p53 response elements. Sites that are bound effectively by bob1 are denoted by black boxes, sites that bind less well are indicated by shaded boxes, and sites that are not bound by bob1 are indicated by the open boxes. The binding specificity of bob2 is distinct from that of bob1 (see text). The rectangles with diagonal stripes denote exons and the elevated arrows represent transcriptional start sites.

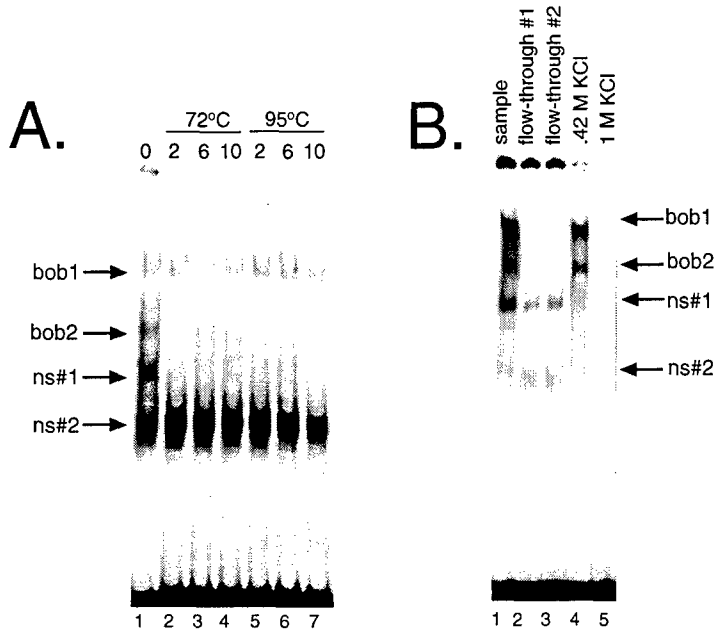

Figure 2. Bob1 is heat resistant wheras bob2 is heat sensitive; both factors bind to SP Sepharose.

(A) EMSA was performed using the bax oligonucleotide as radiolabeled probe and nuclear extract from Hela cells that had been pretreated as indicated.

(B) Nuclear extract from Hela cells was bound to SP Sepharose, washed extensively, and then eluted as indicated. An EMSA was performed using the bax oligonucleotide as radiolabeled probe.

bob1 and bob2 indicate the positions of the two sequence-specific DNA-binding factors, and ns#1 and ns#2 indicate the position of two non-specific bands.

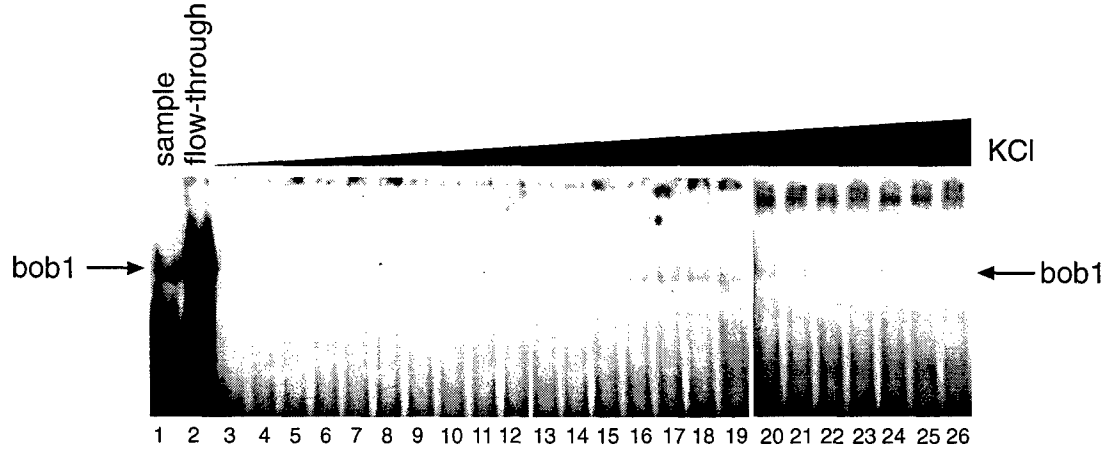

Figure 3.. Bob1 binds to a bax affinity column.

Heat-treated nuclear extract from Hela cells was bound to a bax affinity column, the column was washed, and then fractions were collected upon elution of a gradient from 01-1M KCl. An EMSA was performed using the bax oligonucleotide as radiolabeled probe. The position of the bob1-DNA complex is indicated.

activation by the p53 homologs, p73 and p63 (data not shown). Finally, binding of bob1 and bob2 to DNA is inhibited by HMG-1 whereas the binding of p53 to DNA is enhanced by HMG-1 (data not shown). Preliminary EMSA studies have suggested that the binding of p53 and these nuclear factors may be mutually exclusive, suggesting that the interaction of these factors with a subset of p53 response elements may, indeed, be a mechanism for regulation of p53 target gene selectivity.

Two pieces of evidence argues that bob1 and bob2 are, in fact, distinct. First, bob2 binds to the intronic site from the cyclin G gene and the A site from the IGFBP3 gene whereas bob1 does not (Figure 1B). Second, the binding of bob1 to DNA is heat resistant, whereas that of bob2 is heat sensitive (Figure 2A). Treatment of nuclear extract at either 72°C or 95°C for as little as 2 min causes loss of the bob2-DNA complex without affecting

that of bob1 (Figure 2A). Such heat-inactivated nuclear extract from Hela cells was bound to a DNA affinity column consisting of the *bax* oligonucleotide cross-linked to Sepharose beads. After elution with a gradient of 0.1-1M KCl, bob1 activity was detected in fractions eluted with 0.6-0.7 M KCl, consistent with the high affinity binding of bob1 to the *bax* site that was seen by EMSA (Figure 3). A preliminary purification step is desirable prior to DNA affinity column, primarily to reduce the levels of nucleases present in nuclear extract which can degrade the oligonucleotide that is bound to the Sepharose beads. Several conventional chromatographic media were tested for this purpose. SP Sepharose was chosen as both ns#1 and ns#2 failed to bind to SP Sepharose whereas both bob1 and bob2 were retained and found to elute at 0.42M KCl (Figure 2B). Thus, two novel nuclear factors have been identified that display sequence-specificity for only a subset of p53 genomic binding sites. Preliminary chromatographic studies suggest that purification of these two complexes will be feasible and heat treatment may be used to distinguish between the two factors.

Discussion

Preliminary studies have demonstrated that both bob1 and bob2 are present in nuclear extract derived from Hela cells (Figure 1B). As Hela cells can be grown in suspension, they can be grown in large quantities. The main purification step will involve DNA affinity chromatography using the an oligonucleotide containing the sequence of the *bax* site covalently attached to Sepharose beads. Two non-specific DNA complexes were identified in Hela cell nuclear extracts (Figure 1B). Both of these have been seen to co-purify with the specific factors on a *bax* site affinity column. To clear the extract of these non-specific factors as well as any nucleases which may degrade the DNA attached to the affinity column, a preliminary purification step is necessary. Screening of several conventional chromatographic media has shown that the strong cation exchanger, SP Sepharose, is most suitable (Figure 2B). Both non-specific factors elute with the flow-through on such a column (Figure 2B). Hence a purification scheme will involve preparation of nuclear extract for Hela cells. 12 liters has shown to be a reasonable amount of starting material. The nuclear extract will be diluted to 100 mM KCl and subjected to SP Sepharose chromatography. It has been determined that optimal elution of both bob1 and bob2 occurs at 0.42M KCl. Fractions will be collected and screened for the presence of activity by EMSA. Appropriate fractions will be pooled, dialyzed , and then subjected to *bax* site affinity chromatography. Elution of bob1 from this column has been seen at 0.6-0.7M KCl (Figure 3). Fractions will again be screened by EMSA and then examined by SDS polyacrylamide gel electrophoresis followed by silver-staining. Previous experience with DNA affinity chromatography has shown that there are often three common contaminating proteins that elute in a specific manner from such columns. These are the poly-ADP ribose polymerase (PARP, 116 kd) and the two Ku antigens (70 kd, and 86 kd). Antibodies are available in our laboratory to discern whether these proteins are eluted in the same fractions as bob1 and bob2 and to verify that any protein bands that are identified do not reflect any of these contaminants. It is expected that after the DNA affinity chromatography step, the factors will be sufficiently pure to allow excision of bands from an SDS polyacrylamide gel and subsequent microsequencing. If the preparation still contains multiple protein bands, further purification will be necessary and a variety of conventional chromatographic procedures will be attempted. If database searches determine the binding factor to be novel, then degenerate oligonucleotides will be synthesized according to the amino acid sequence and used in a polymerase chain reaction

of a HeLa cell cDNA library. The resulting PCR product subsequently will be used as a probe to screen the cDNA library for the complete clone of the protein.

One problem with this approach is that both bob1 and bob2 will co-purify using this scheme. To address this, the differing response of each of these factors to heat will be exploited. Bob1 has been shown to be heat resistant whereas bob2 is heat sensitive (Figure 2A). This differential response to heat will be used to attempt to purify bob1 separately from bob2. Nuclear extract that has been treated at 95°C for 5 min has been subjected to DNA affinity chromatography. Bob1, but not bob2, complexes are seen in fractions subjected to EMSA from such a column (Figure 3). Hence, purifications will be performed with both heat-treated and untreated nuclear extracts. The final preparations from each of these purifications will be compared to assign particular protein bands to either the bob1 or bob2 factor.

Adherence to Statement of Work

Technical Objective #1		
Task 1:	Months 1-2	Completed
Task 2:	Months 3-6	Completed
Task 3:	Months 7-12	Completed
Technical Objective # 2		
Task 4:	Months 6-12	Completed
Task 5:	Months 13-18	In progress
Task 6:	Months 11-14	In progress
Task 7:	Months 15-20	In progress
Technical Objective #3		
Tasks 8-11:	Months 19-36	Pending

It was hoped that by the start of Year 3, sufficient material would be available for microsequencing. Although this goal was not achieved, it is expected that purification will be completed within 2 months at the latest.

KEY RESEARCH ACCOMPLISHMENTS

- identification of two nuclear factors which bind DNA in a sequence-specific manner
- these two nuclear factors bind to only a subset of genomic p53 response elements
- the two nuclear factors are distinct based on thermal sensitivity
- preliminary studies suggest purification of each of these factors is feasible

REPORTABLE OUTCOMES

Manuscripts

Thornborrow, E.C. and Manfredi, J.J. (1999) One mechanism for cell-type specific regulation of the *bax* promoter by the tumor suppressor p53 is dictated by the p53 response element. J. Biol. Chem. **274**: 33747-33755.

Abstracts

Resnick-Silverman, L., St. Clair, S., Thornborrow, E., Maurer, M., Meng, J., Ream, A., and Manfredi, J.J. (1999) Target gene selection by p53 is regulated by multiple mechanisms. ICGEB Workshop: "p53: Twenty Years On" (Trieste, Italy).

Presentations

Target gene selection by p53 is regulated by multiple mechanisms
ICGEB Workshop: p53: Twenty Years On, Trieste, Italy
May 21, 1999

Determinants of the cellular response to the tumor suppressor p53
San Raffaele Scientific Institute, Milan , Italy
May 24, 1999

Funding applied for based on work supported by this award

Title of Project:	Determinants of cellular responses to p53
Sponsoring agency:	National Institutes of Health/National Cancer Institute
Number:	1 R01 CA86001-01
Grant type:	Research
Status:	Principal Investigator
Percent Effort:	25%
Total Project Period:	04/01/00-03/31/05
Total direct costs:	$875,000

This application was reviewed by the Pathology B Scientific Review Group and received a priority score of 263 and a percentile of 55.1.

CONCLUSIONS

Two cellular factors, named bob1 and bob2, have been identified which bind in a sequence-specific manner to a subset of p53 response elements. Preliminary studies suggest that the binding of these factors and p53 may be mutually exclusive. Occupancy of particular p53 binding sites but not others by one of these factors may inhibit the ability of p53 to activate expression of some target genes but not others. To address this possibility, the two factors will be biochemically purified, their respective cDNAs cloned, and studies will be performed to directly address whether these factors inhibit the ability of p53 to activate expression of a subset of its target genes.

The long term goal is to determine the relevance of these nuclear factors as well as the high mobility group protein HMG-1 in human breast cancer. Human breast tumor samples would be screened for alterations in the expression of these factors which affect the DNA binding activity of p53. The identification of proteins which regulate wild-type p53 is an important focus for breast cancer research since the regulation, mechanism of action, and metabolism of such proteins would be central to our understanding of breast cancer and the aberrant expression of such proteins would represent novel important mechanisms of carcinogenesis.

APPENDICES

THE JOURNAL OF BIOLOGICAL CHEMISTRY
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One Mechanism for Cell Type-specific Regulation of the *bax* Promoter by the Tumor Suppressor p53 Is Dictated by the p53 Response Element*

(Received for publication, March 11, 1999, and in revised form, September 2, 1999)

Edward C. Thornborrow and James J. Manfredi‡

From the Derald H. Ruttenberg Cancer Center, Mount Sinai School of Medicine, New York, New York 10029

Key to the function of the tumor suppressor p53 is its ability to activate the transcription of its target genes, including those that encode the cyclin-dependent kinase inhibitor p21 and the proapoptotic Bax protein. In contrast to Saos-2 cells in which p53 activated both the *p21* and *bax* promoters, in MDA-MB-453 cells p53 activated the *p21* promoter, but failed to activate the *bax* promoter. Neither phosphorylation of p53 on serines 315 or 392 nor an intact C terminus was required for p53-dependent activation of the *bax* promoter, demonstrating that this differential regulation of *bax* could not be explained solely by modifications of these residues. Further, this effect was not due to either p73 or other identified cellular factors competing with p53 for binding to its response element in the *bax* promoter. p53 expressed in MDA-MB-453 cells also failed to activate transcription through the p53 response element of the *bax* promoter in isolation, demonstrating that the defect is at the level of the interaction between p53 and its response element. In contrast to other p53 target genes, like *p21*, in which p53-dependent transcriptional activation is mediated by a response element containing two consensus p53 half-sites, activation by p53 of the *bax* element was mediated by a cooperative interaction of three adjacent half-sites. In addition, the interaction of p53 with its response element from the *bax* promoter, as compared with its interaction with its element from the *p21* promoter, involves a conformationally distinct form of the protein. Together, these data suggest a potential mechanism for the differential regulation of p53-dependent transactivation of the *bax* and *p21* genes.

The tumor suppressor protein p53 is an important regulator of cellular growth. The *p53* gene is mutated in the majority of human cancers (1, 2), suggesting that loss of p53 may play an important causative role in oncogenesis. The p53 protein has been implicated in several diverse growth-related pathways, including apoptosis, cell cycle arrest, and senescence (3–5). The ability of p53 to function as a sequence-specific DNA-binding protein appears to be central to the function of p53 as a tumor suppressor (6, 7). At its N terminus, the p53 protein contains a potent transcriptional activation domain (8) that is linked to a central core domain that mediates sequence-specific DNA binding (9–11). Both of these domains have been shown to be important for p53-mediated growth suppression (12). The importance of the DNA binding domain is further highlighted by the fact that the major mutational hot spots from human cancers are found in this domain (13), and several of these mutations have been shown to abolish the ability of p53 to function as a transcriptional activator (14–16).

A DNA consensus sequence through which p53 binds and activates transcription has been identified. This sequence consists of two palindromic decamers of 5′-RRRCWWGYYY-3′ (where R is a purine, Y is a pyrimidine, and W is an adenine or thymine) separated by 0–13 bp,[1] forming four repeats of the pentamer 5′-RRRCW-3′ alternating between the top and bottom strands of the DNA duplex (17–19). This arrangement is consistent with the notion that p53 binds DNA as a homotetramer (20–23). Through sequences similar to this consensus, p53 has been shown to activate the transcription of many genes, including *bax*, *p21*, *mdm2*, *gadd45*, *IGF-BP3*, and *cyclin G* (24–31). Data are consistent with a model in which DNA damage leads to the phosphorylation of p53 as well as the subsequent stabilization of p53 and activation of its DNA binding capability (32–35). Consequently, p53-mediated transcription of its target genes increases. When compared with alternate p53 targets, such as the cyclin-dependent kinase inhibitor *p21*, evidence suggests that the *bax* gene is differentially regulated by p53. Several tumor-derived p53 mutants have been identified that are capable of activating transcription through the promoter of the *p21* gene but not through the *bax* promoter (36–39). This has been correlated with an inability of the mutants both to bind the p53 response element of the *bax* promoter and to trigger apoptosis (36, 38, 39). Such studies with these tumor-derived p53 mutants suggest that a failure in the ability of p53 to activate the *bax* gene may play an important role in tumor formation and progression. As such, a complete understanding of the transcriptional regulation of the *bax* promoter by p53 may yield important information relevant to our understanding of tumorigenesis.

Previous studies have demonstrated that the *bax* promoter is differentially regulated by wild-type p53 in a cell type-specific manner (40). Here the osteosarcoma Saos-2 and the breast carcinoma MDA-MB-453 cell lines were used as a model system to explore the potential mechanisms for this differential regulation. In the Saos-2 cell line, transfected wild-type p53 effectively activated transcription through both the *p21* and *bax* promoters. In contrast, p53 expressed in the MDA-MB-453 cell line was capable of activating transcription through the *p21* promoter as well as the p53 response elements of the *p21*, *cyclin G* and *cdc25C* promoters but failed to do so through

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[1] The abbreviations used are: bp, base pair(s); mAb, monoclonal antibody.

either the *bax* promoter or the isolated p53 response element derived from the *bax* promoter. Neither p53 phosphorylation at serine 315 or serine 392 nor an intact C terminus was required for activation of the *bax* promoter, demonstrating that the observed defect in MDA-MB-453 cells could not be explained solely by modifications of these residues. In addition, neither the p53 homolog p73 nor other cellular factors that are capable of binding the p53 response element of the *bax* promoter explained the differential regulation of the *bax* promoter. Detailed analysis of the interaction of p53 with the *bax* promoter, however, demonstrated that unlike other well characterized p53 response elements, like that of the *p21* gene, in which p53-dependent transcriptional activation is mediated by a response element containing two consensus p53 half-sites, the response element of the *bax* promoter consists of three adjacent half-sites that cooperate to bring about complete activation by p53. In addition, it appears that p53 exists in a distinct conformation when bound to its response element from the *bax* promoter as compared with when it is bound to the 5′-response element of the *p21* promoter. Together, these data suggest a potential mechanism for the cell type-specific differential regulation of *bax* by p53.

MATERIALS AND METHODS

Oligonucleotides—For use in electrophoretic mobility shift assays and for subsequent cloning into luciferase reporter plasmids, complementary single-stranded oligonucleotides were annealed to produce double-stranded oligonucleotides with the indicated sequences: Bax, AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGCGTGGGCTATATTGTAGCGAATT; OligoA, AATTCGGTACCTCACAAGTTAGAGACAAGCCTGCTAGCGAATT; OligoB, AATTCGGTACCAGACAAGCCTGGGCGTGGGCGCTAGCGAATT; OligoC, AATTCGGTACCAGACAAGCCTTTTACGGGGCTATATTGCTAGCGAATT; OligoAB, AATTCGGTACCTCACAAGTTAGAGACAAGCCTGGGCGTGGGCGCTAGCGAATT; OligoAC, AATTCGGTACCTCACAAGTTAGAGACAAGCCTTTTACGGGGCTATATTGCTAGCGAATT; OligoBC, AATTCGGTACCAGACAAGCCTGGGCGTGGGCTATATTGCTAGCGAATT; p21–5′, AATTCGGTACCGAACATGTCCCAACATGTTGGCTAGCGAATT; p21–3′(2x), AATTCGGTACCGAAGAAGACTGGGCATGTCTGAAGAAGACTGGGCATGTCTGCTAGCGAATT; Cyclin G-AATTCGAGCTCCAAGGCTTGCCCGGGCAGGTCTGGGTACCGAATT; Cdc25C(2x), AATTCGGTACCGGGCAAGTCTTACCATTTCCAGAGCAAGCACGCTAGCAGGCCTGTGCTTGCTCTGGAAATGGTAAGACTTGCCCAGATCTAATATTG; and Sens-1, TCGAAGAAGACGTGCAGGGACCCTCGA.

Plasmids—The expression plasmids pCMV-p53^{wt}, pCMV-p53^{V143A}, and pCMV-p53^{S392A}, originally referred to as pC53-SN3 (41), pC53-SCX3 (14), and pCMVhup53ala392 (42), respectively, encode the indicated human p53 protein under the control of the cytomegalovirus promoter. The expression plasmid pCMV-p53$^{\Delta370-393}$, originally referred to as pCB6+p53Δ370 (43), encodes p53, under the control of the cytomegalovirus promoter, with a point mutation introducing a stop codon at amino acid 370. The expression plasmids pB-p53^{S315A} and pB-p53^{S315D}, originally referred to as Bhup53ala315 (42) and Bhup53asp315 (42), respectively, encode the indicated human p53 protein under the control of the human B-actin promoter. The expression vector pCMV-p73α encodes wild-type p73 under the control of the cytomegalovirus promoter (44). The luciferase reporter plasmid p21P contains the 2.4-kilobase *Hin*dIII fragment from the *p21* promoter cloned into the pGL2-Basic vector (45). The luciferase reporter plasmid pBax contains the 370-bp *Sma*I/*Sac*I fragment from the *bax* promoter cloned into the pGL3-Basic vector (29). The following synthetic double-stranded oligonucleotides were digested with *Kpn*I and *Nhe*I and cloned into pGL3-E1bTATA (46), which also had been double-digested with *Kpn*I and *Nhe*I to produce pTATA vectors with corresponding names: Bax, OligoA, OligoB, OligoC, OligoAB, OligoBC, OligoAC, p21–5′, p21–3′ (x2), Cyclin G, and Cdc25C (x2) (46).

Cell Lines—The osteosarcoma Saos-2 cell line and the breast carcinoma MDA-MB-453 cell line were maintained in a humidified tissue culture incubator at 37 °C with 5% CO_2. Saos-2 cells were grown in Dulbecco's modified Eagle's medium, containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. MDA-MB-453 cells were grown in RPMI ~~media~~, containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml insulin.

Transfections—Unless otherwise indicated, 1×10^5 cells were seeded into 35-mm plates. Cells were transfected 24 h later using the DOTAP liposomal transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. Cellular lysates were prepared 48 h post-transfection, total protein concentration was determined by protein assay (Bio-Rad), and luciferase assays were quantitated using a commercially available kit (Promega) and a TD-20e Luminometer (Turner).

Nuclear Extracts—All procedures were conducted at 4 °C. For each 100-mm dish, cells were washed three times with 5 ml of phosphate-buffered saline. Cells then were scraped into 500 μl of lysis buffer (20 mM HEPES, pH 7.5, 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 50 μM leupeptin, and 50 μg/ml aprotinin) and centrifuged at 500 × *g* for 5 min. Pellets were resuspended in 200 μl of nuclear extraction buffer (lysis buffer containing 500 mM NaCl) and incubated end-over-end for 60 min. Samples were centrifuged at 18,000 × *g* for 10 min. Nuclear extracts were aliquoted, quick-frozen in liquid nitrogen, and stored at −70 °C.

Electrophoretic Mobility Shift Assays—Purification of human p53 protein and electrophoretic mobility shift assays using this purified p53 were conducted as described previously (46). In brief, Sf9 cells that were infected with recombinant baculovirus expressing His-tagged p53 were lysed in 20 mM HEPES, pH 7.4, containing 20% glycerol, 10 mM NaCl, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 50 μM leupeptin, and 50 μg/ml aprotinin (Buffer L). Nuclei were pelleted by centrifugation at 2300 rpm and then resuspended in Buffer L containing 500 mM NaCl. Extracts were diluted to 100 mM NaCl with Buffer L, applied to a 0.5-ml ~~Ni-NTA~~ Nickel-nitriloacetic acid agarose column (Qiagen) that was equilibrated with 20 mM HEPES containing 100 mM NaCl and eluted with 200 mM imidazole containing 10 mM HEPES, pH 7.4, and 5 mM NaCl. Fractions of 0.5 ml were collected, dialyzed against 10 mM HEPES, pH 7.4, 5 mM NaCl, 0.1 mM EDTA, 20% glycerol, and 1 mM dithiothreitol, aliquoted, and stored at −70 °C.

Purified p53 protein or nuclear extract was incubated with 3 ng of radiolabeled double-stranded oligonucleotide and hybridoma supernatant where appropriate in a total volume of 30 μl of DNA binding buffer, containing 20 mM $MgCl_2$, 2 mM spermidine, 0.7 mM dithiothreitol, 1 mg/ml bovine serum albumin, and 25 μg/ml poly[d(I-C)] for 30 min at room temperature. Samples were loaded on a native 4% acrylamide gel and electrophoresed in 0.5× TBE at 225 V for 2 h at 4 °C. Gels were dried and exposed to Kodak XAR-5 film using an intensifying screen at −70 °C. Bands were scanned and quantitated using the Molecular Analyst Imaging Densitometer (Bio-Rad).

SDS-Polyacrylamide Gel Electrophoresis and Western Blot—Cells were lysed in 150 mM NaCl, 50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 50 μM leupeptin, and 10 μg/ml aprotinin. The protein concentration of each sample was determined using the Bio-Rad Protein Assay. Samples containing equal amounts of protein were electrophoresed in a 10% polyacrylamide gel. Following electrophoresis, protein was transferred to nitrocellulose and probed with a 1:1 mixture of the anti-p53 mouse monoclonal antibodies 1801 and 421. The secondary antibody was a horseradish peroxidase-conjugated goat anti-mouse IgG, and the signal was detected by the enhanced chemiluminescence method (Amersham Pharmacia Biotech).

RESULTS

Wild-type p53 Fails to Activate Transcription through the p53 Response Element of the bax Promoter in the Breast Carcinoma MDA-MB-453 Cell Line—Wild-type p53 expressed in the breast carcinoma MDA-MB-453 cell line is unable to activate transcription through the *bax* promoter or through the isolated p53 response element of the *bax* promoter (Figs. 1*A* and 2*A*). Luciferase reporter plasmids containing either the *p21* promoter or the *bax* promoter were transfected into the p53-negative Saos-2 or MDA-MB-453 cell line with pCMV vector, increasing amounts of a plasmid expressing wild-type p53, or a plasmid expressing the mutant p53^{V143A}. In the Saos-2 cell line, wild-type p53 effectively activated transcription of reporter constructs containing either the *p21* or *bax* promoters. In contrast, wild-type p53 expressed in the MDA-MB-453 cell line, although still capable of activating transcription of a reporter containing the *p21* promoter, failed to activate transcription through a construct containing the *bax* promoter (Fig. 1*A*). Western blots

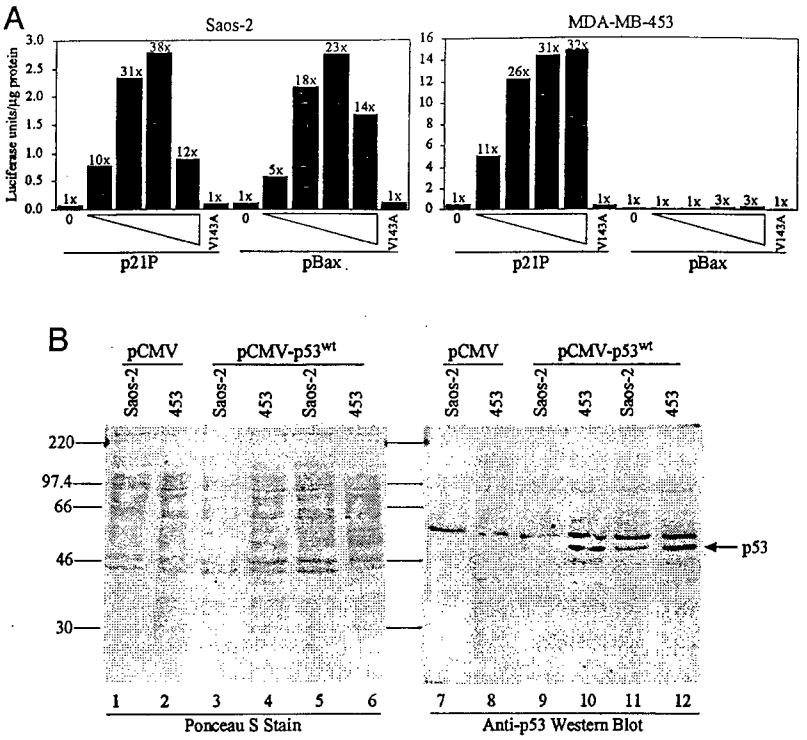

FIG. 1. **Expression of wild-type p53 in MDA-MB-453 cells fails to activate transcription through the *bax* promoter.** *A*, Saos-2 and MDA-MB-453 cells were transfected as described under "Materials and Methods" with 2 μg of the indicated reporter constructs in the presence of 0, 5, 50, 100, or 200 ng of pCMV-p53^{wt} or 50 ng of pCMV-p53^{V143A}. 48 h post transfection cells were lysed and assayed for total protein and luciferase activity as described under "Materials and Methods." Appropriate amounts of the vector pCMV were added to each transfection mixture to maintain a constant level of total plasmid DNA of 2.2 μg/sample. The indicated values are the average of three independent experiments each performed in duplicate. The *numbers* above each *bar* indicate the fold activation for each reporter construct observed with pCMV-p53^{wt} or pCMV-p53^{V143A} as compared with pCMV. *B*, 1×10^6 cells of either Saos-2 (*lanes 7, 9,* and *11*) or MDA-MB-453 (*lanes 8, 10,* and *12*) were seeded in 100-mm plates and subsequently transfected with 10 μg of either empty pCMV (*lanes 7* and *8*) or pCMV-p53^{wt} (*lanes 9–12*). 48 h post transfection cells were lysed and assayed for p53 expression levels by Western blot (*lanes 7–12*) as described under "Materials and Methods." Following immunodetection the blot was stained with Ponceau S to confirm that equal amounts of protein were loaded in each lane (*lanes 1–6*). Each lane contains 60 μg of total protein, and each lane represents an independent transfection.

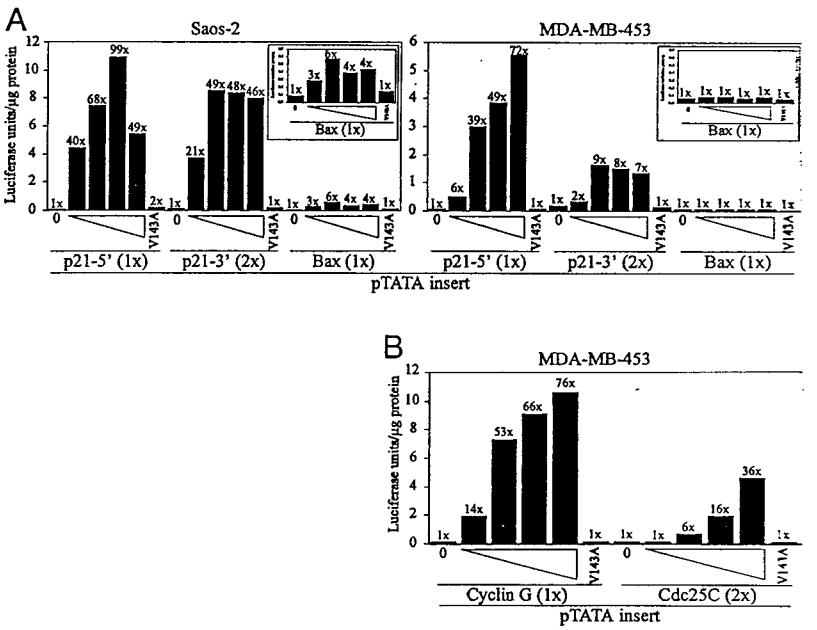

FIG. 2. **Expression of wild-type p53 in MDA-MB-453 cells fails to activate transcription through a 37-bp element of the *bax* promoter.** Saos-2 (*A*) and MDA-MB-453 (*A* and *B*) cells were transfected as described under "Materials and Methods" with 2 μg of the indicated reporter constructs in the presence of 0, 5, 50, 100, or 200 ng of pCMV-p53^{wt} or 50 ng of pCMV-p53^{V143A}. 48 h post transfection cells were lysed and assayed for total protein and luciferase activity as described under "Materials and Methods." Appropriate amounts of the vector pCMV were added to each transfection mixture to maintain a constant level of plasmid DNA of 2.2 μg/sample. The Bax (1x) data are enlarged for clarity (*inset A*). The plots shown in the *insets* have the same scale. The indicated values are the average of three independent experiments each performed in duplicate. The *numbers* above each *bar* indicate the fold activation for each pTATA construct observed with pCMV-p53^{wt} or pCMV-p53^{V143A} as compared with pCMV.

demonstrated that p53 was expressed to equivalent levels in the two cell lines (Fig. 1*B*, compare *lane 9* with *lane 10* and *lane 11* with *lane 12*), if not slightly higher in MDA-MB-453 (Fig. 1*B*, *lanes 10* and *12*), suggesting that the failure of p53 to activate transcription through the *bax* promoter is not due to decreased levels of p53 protein expression.

To determine whether the isolated p53 response element of the *bax* promoter was sufficient for this differential effect, synthetic oligonucleotides corresponding to the p53 response elements of the *p21* and *bax* promoters were cloned into the pGL3-E1bTATA luciferase reporter vector, upstream from the minimal adenovirus *E1b* promoter. Each reporter construct again was transfected into either the Saos-2 or MDA-MB-453 cell line with pCMV vector, increasing amounts of the wild-type

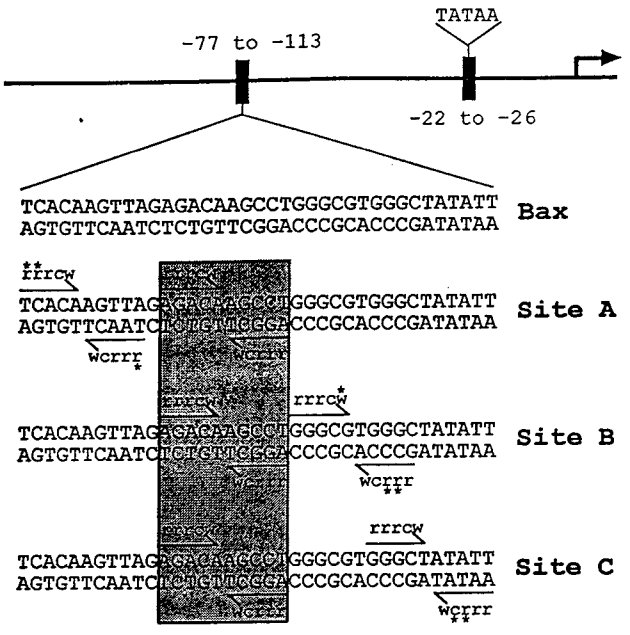

FIG. 3. **Schematic of the p53 response element of the human *bax* promoter.** The previously identified p53 response element of the *bax* promoter is located at −113 to −77 from the transcriptional start site. Based on the p53 consensus binding site, there exists, within this 37-bp sequence, three potential, overlapping p53 binding sites. These putative binding sites are labeled Site A (−113 to −93), Site B (−102 to −83), and Site C (−102 to −77). The *arrows* indicate the four quarter sites that constitute each proposed p53 binding site. The p53 consensus sequence is indicated above each *arrow* with *R* representing purine, *Y* representing pyrimidine, and *W* representing either an adenine or thymine base. Bases in the *bax* sequence that vary from this consensus are indicated by *asterisks*. The perfect half-site shared by each potential binding site is highlighted by the *gray box*. The position of the TATA box for the *bax* promoter (−22 to −26) also is indicated.

p53 expression plasmid, or the mutant $p53^{V143A}$ expression plasmid. In the Saos-2 cell line, wild-type p53 effectively activated transcription of constructs containing either the 5′ or the 3′ p53 response elements from the *p21* promoter (Fig. 2*A*), as well as a construct containing the p53 response element of the *bax* promoter (Fig. 2*A*, *inset*). As observed with the promoter constructs, wild-type p53 expressed in the MDA-MB-453 cell line failed to activate transcription via an *E1b* reporter plasmid containing the p53 response element of the *bax* promoter (Fig. 2*A*, *inset*), whereas activating reporters containing either the 5′ or the 3′ element of the *p21* promoter (Fig. 2*A*). Expression of wild-type p53 in MDA-MB-453 cells also activated transcription of reporters containing the p53 response elements of the *cyclin G* and *cdc25C* genes (Fig. 2*B*). Thus, the defect in p53-dependent transcriptional activation of the *bax* promoter appears to be at the level of the interaction of p53 with its response element.

The p53 Response Element of the bax Promoter Consists of Overlapping Binding Sites for p53—The data presented in Figs. 1 and 2 demonstrate that in MDA-MB-453 cells there is a defect in wild-type p53-dependent activation via the 37-bp p53 response element of the *bax* promoter, as compared with the 5′ p53 response element of the *p21* promoter. To understand the molecular mechanism mediating this differential regulation of the p53 response elements, the interaction between p53 and its response element from the *bax* promoter was examined in detail by electrophoretic mobility shift assays. Previous studies localized the p53 response element of the *bax* promoter to a 37-bp region at −113 to −77 from the start site of transcription (29). An examination of the nucleotide sequence of this 37-bp element revealed three potential p53 binding sites, termed Site A, Site B, and Site C (Fig. 3), that correspond to the consensus site for p53 binding (17–19). Site A consists of the first 21 bp of the 37-bp response element, with two potential p53 half-sites separated by a 1-bp insert. The first half-site contains three bases that vary from the consensus (two purine-to-pyrimidine changes in the first quarter-site and one in the second quarter-site). The second half-site of Site A matches the consensus sequence in all 10 bases. Site B consists of 20 bp including this same "consensus" half-site and a second half-site downstream, separated by no intervening sequences. Site B diverges from the consensus at three bases (the A/T is a G in the last position of the third quarter-site, and there are two purine-to-pyrimidine changes in the fourth quarter-site). Site C consists of 26 bp and includes the same half-site noted in Sites A and B, separated from a second half-site by a 6-bp insert. Site C contains two variations from the consensus sequence (a C to A change and a purine-to-pyrimidine change both in the fourth quarter site). Of note is the spatial relationship of these three potential p53 binding sites. The three sites overlap one another with the consensus half-site (−102 to −93) common to each. Because of this shared half-site, the binding of p53 to one site excludes its simultaneous binding to either of the other sites. Therefore, if one assumes that p53 binds as a tetramer (20–22), then only one site can be occupied at any given time.

To identify which of these putative binding sites are responsible for the interaction between p53 and the *bax* promoter, synthetic double-stranded oligonucleotides were constructed to model each site (Table I). The Bax oligonucleotide contained the complete 37-bp p53 response element from the *bax* promoter. Oligo A contained the 21 bp corresponding to Site A, whereas Oligo B contained the 20 bp corresponding to Site B. Oligo C consisted of the 26 bp corresponding to Site C; however, because of the sequence overlap between Sites B and C the 6 bp separating the two half-sites in Site C were scrambled to abolish any potential contribution from Site B. Each oligonucleotide contained identical flanking sequences that allowed for its subsequent cloning into a luciferase reporter plasmid. The relative affinities of these oligonucleotides for p53 were assessed by electrophoretic mobility shift assay. Purified p53 bound the labeled Bax oligonucleotide containing the entire 37-bp p53 response element (Fig. 4*A*, *lane 1*), and this binding was effectively competed by an excess of the same, unlabeled oligonucleotide (Fig. 4*A*, *lanes 2–4*). Unlabeled Oligo A, Oligo B, and Oligo C also successfully competed for p53 binding (Fig. 4*A*, *lanes 5–7*, *8–10*, and *11–13*). For comparison, an unrelated control oligonucleotide, Sens-1, was unable to compete for p53 binding (Fig. 4,*A*, *lanes 14–16*, and *B*), demonstrating that the binding of p53 to Oligos A, B, and C is specific. In each case, however, the binding of p53 to the isolated sites was weaker than that observed with the entire 37-bp response element (Fig. 4*B*). These data suggest the possibility that in the context of the entire p53 response element of the *bax* promoter there is a cooperative interaction between the overlapping p53 binding sites that allows for enhanced p53 binding.

The ability of purified p53 to directly bind to these oligonucleotides in electrophoretic mobility shift assays was then examined. A labeled oligonucleotide corresponding to the 5′ p53 response element of the *p21* promoter was used as a positive control for p53 binding (Fig. 5, *lanes 1–3*). The *p21*-5′ oligonucleotide was bound by p53 and was effectively supershifted by mAb 1801, a p53 N-terminal-specific monoclonal antibody (Fig. 5, *lane 2*). In addition, the labeled Bax oligonucleotide, corresponding to the entire p53 response element of *bax*, as well as those corresponding to Site A, Site B, and Site C were also bound by purified p53 (Fig. 5, *lanes 4*, *7*, *10*, and *13*) and were supershifted by mAb 1801 (Fig. 5, *lanes 5*, *8*, *11*, and *14*). This binding, however, was weaker than that observed with the

TABLE I
Synthetic oligonucleotides used in electrophoretic mobility shift assays and transfection assays

Name of oligonucleotide	Nucleotide sequence[a]
Bax	5′-aattcggtacc**TCACAAGTTAGAGACAAGCCTGGGCGTGGGCTATATT**gtagcgaatt-3′
Oligo A	5′-aattcggtacc**TCACAAGTTAGAGACAAGCCT**gctagcgaatt-3′
Oligo B	5′-aattcggtacc**AGACAAGCCTGGGCGTGGGC**gctagcgaatt-3′
Oligo C	5′-aattcggtacc**AGACAAGCCT**tttacg**GGGCTATATT**gctagcgaatt-3′
Oligo AB	5′-aattcggtacc**TCACAAGTTAGAGACAAGCCTGGGCGTGGGC**gctagcgaatt-3′
Oligo AC	5′-aattcggtacc**TCACAAGTTAGAGACAAGCCT**tttacg**GGGCTATATT**gctagcgaatt-3′
Oligo BC	5′-aattcggtacc**AGACAAGCCTGGGCGTGGGCTATATT**gctagcgaatt-3′
p21–5′	5′-aattcggtacc**GAACATGTCCCAACATGTTG**gctagcgaatt-3′

[a] The bold capital letters represent the sequences taken from the *bax* and *p21* promoters. Bases that participate in the formation of potential p53 binding sites are indicated by underlining. The lowercase letters indicate sequences not derived from either the *bax* or *p21* promoters.

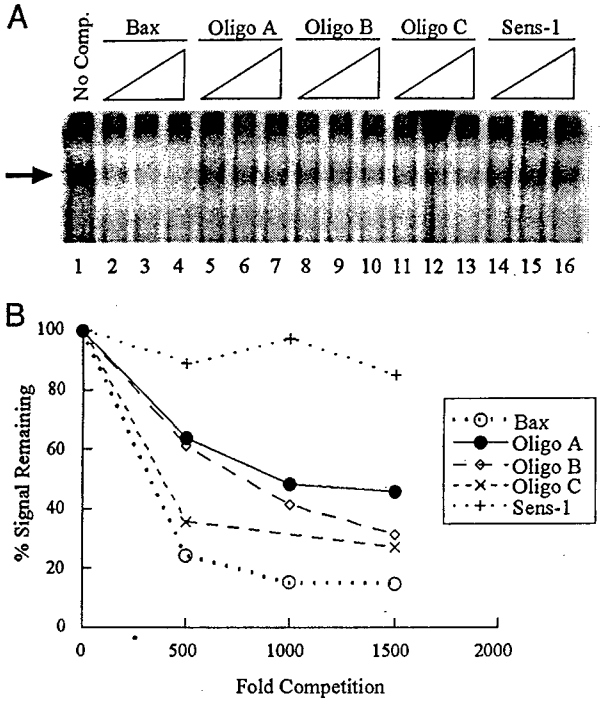

FIG. 4. **The p53 response element of the *bax* promoter contains three overlapping p53 binding sites.** *A*, an electrophoretic mobility shift assay was performed using the Bax oligonucleotide as radiolabeled probe. 50 ng of purified p53 was incubated with 3 ng of the probe alone (*lane 1*) or in the presence of a 500- (*lanes 2, 5, 8, 11,* and *14*), 1000- (*lanes 3, 6, 9, 12,* and *15*), or 1500-fold (*lanes 4, 7, 10, 13,* and *16*) molar excess of the indicated unlabeled competitors. The Sens-1 oligonucleotide (*lanes 14–16*) was used as a nonspecific control. The *arrow* indicates the position of the p53-DNA complexes. Bands were quantitated by densitometry and expressed as a percentage of the no competition signal (*lane 1*) (*B*). The 1000x point of the Oligo C competition (*lane 12*) was not included because of an artifactual streak in the lane that interfered with quantitation.

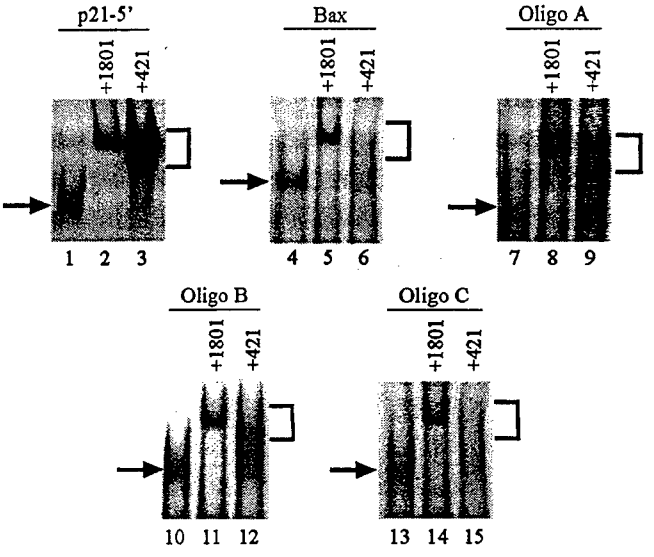

FIG. 5. **Monoclonal antibody 421 enhances the binding of p53 to the *p21* site but inhibits the binding of p53 to the *bax* sites.** An electrophoretic mobility shift assay was performed, incubating either 5 ng (*lanes 1–3*) or 50 ng (*lanes 4–15*) of purified p53 with 3 ng of the indicated radiolabeled probes in the absence (*lanes 1, 4, 7, 10,* and *13*) or presence of monoclonal antibodies 1801 (*lanes 2, 5, 8, 11,* and *14*) or 421 (*lanes 3, 6, 9, 12,* and *15*). The *arrows* indicate the positions of the p53-DNA complexes, and the *brackets* indicate the positions of the supershifted antibody-p53-DNA complexes.

p21-5′ site, requiring approximately 10-fold more p53 to generate a detectable band shift.

Previously our laboratory reported two distinct classes of p53 binding sites based on their responses to the C-terminal-specific mAb 421 (46). p53 binding to one class of sites, which includes the *p21*-5′ site, is enhanced in the presence of mAb 421, whereas binding to the second class of sites is inhibited by mAb 421. Confirming our original observation, p53 binding to the *p21*-5′ site was enhanced in the presence of mAb 421 (Fig. 5, *lane 3*). Binding of p53 to the Bax oligonucleotide as well as to Oligo C, however, was inhibited in the presence of mAb 421 (Fig. 5, *lanes 6* and *15*). The binding of p53 to Oligos A and B displayed an intermediate phenotype, in which mAb 421 failed to effectively supershift the p53-oligonucleotide complexes and failed to enhance p53 binding to the oligonucleotides (Fig. 5, *lanes 9* and *12*). In either case, the data are consistent with the notion that binding to each of the *bax* sites as compared with the *p21*-5′ site may require a conformationally distinct form of p53.

Overlapping, Low Affinity p53 Binding Sites Synergize for Complete p53-dependent Transactivation through the p53 Response Element of the bax Promoter—The Bax oligonucleotide as well as Oligo A, Oligo B, and Oligo C were cloned into the pGL3-E1bTATA luciferase reporter vector upstream from the adenovirus minimal *E1b* promoter. Each reporter construct was transfected with the pCMV empty vector, a plasmid expressing wild-type p53, or a plasmid expressing the temperature-sensitive $p53^{V143A}$ mutant into the p53-negative Saos-2 cell line (Fig. 6). At 37 °C the $p53^{V143A}$ mutant fails to activate transcription through p53-responsive promoters. At 32 °C, however, this mutant adopts a wild-type conformation and has been shown to activate some p53-responsive promoters (such as *p21*) but not others (such as *bax*) (36, 38). At 37 °C, wild-type p53 activated transcription through the complete 37-bp response element of the *bax* promoter (Fig. 6*A*). In addition, wild-type p53 activated transcription through Oligo B; however, this activation was significantly lower than that observed with the complete response element (21-fold compared with 67-fold). Although Oligos A and C both showed sequence-specific binding to p53 in an electrophoretic mobility shift assay (Fig. 4), p53 failed to activate transcription, to any significant

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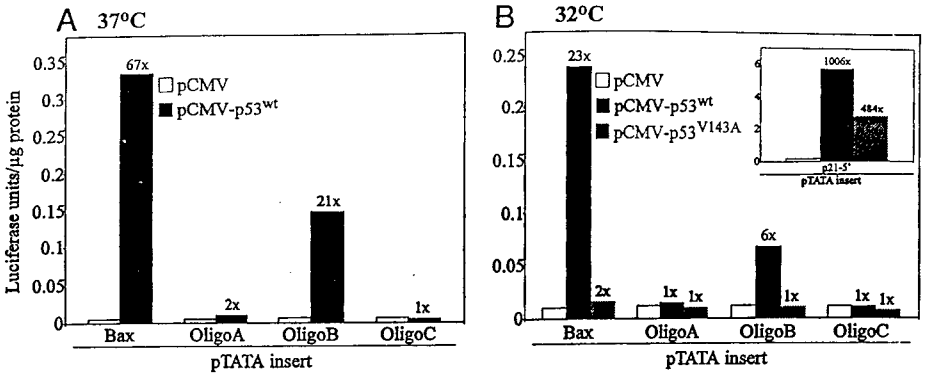

FIG. 6. Site B is sufficient to confer p53-dependent transactivation, but the level of transactivation is lower than that observed with the complete 37-bp response element. Saos-2 cells were transfected as described under "Materials and Methods" with 2 μg of the indicated reporter constructs and 50 ng of either empty pCMV (*white bars*), the wild-type p53 expression vector pCMV-p53^{wt} (*black bars*), or the temperature-sensitive p53 expression vector pCMV-p53^{V143A} (*gray bars*). Cells were maintained either at 37 °C (*A*) or shifted to 32 °C 24 h prior to lysis (*B*). Luciferase activity and total protein levels were assayed as described under "Materials and Methods." The pTATA-p21–5′ reporter construct (*B inset*) was used as a positive control for the pCMV-p53^{V143A} expression vector. The indicated values are the averages of three independent experiments each performed in duplicate. The *numbers* above each *bar* indicate the fold activation for each pTATA construct observed with pCMV-p53^{wt} or pCMV-p53^{V143A} as compared with pCMV.

degree, through either sequence (Fig. 6*A*, 2- and 1-fold, respectively). The same pattern of activation was observed with wild-type p53 at 32 °C (Fig. 6*B*). Similar to observations made with the *bax* promoter (36, 38), the temperature-sensitive p53^{V143A} mutant at 32 °C failed to activate transcription through any of the isolated p53 binding sites of the *bax* promoter (Fig. 6*B*, *gray bars*). The p53^{V143A} mutant, however, did successfully activate transcription through the *p21*-5′ response element inserted into the same pGL3-E1bTATA reporter vector (Fig. 6*B*, *inset*).

The transfection data demonstrate that Site B can mediate p53-dependent activation but that the level of activation conferred by this sequences is one-third of that observed with the complete 37-bp response element. To analyze which additional sequences in the 37-bp element are necessary for full activation, another set of synthetic double-stranded oligonucleotides was constructed (Table I). Oligo AB contained the 31 bp that correspond to the overlapping Sites A and B. Oligo AC consisted of the 37-bp response element; however, the 6 bp separating the two half-sites in Site C were scrambled to abolish any potential contribution from Site B. Oligo BC contained the 30 bp corresponding to the overlapping Sites B and C. Again, each oligonucleotide contained identical flanking sequences that allowed for its subsequent cloning into a luciferase reporter plasmid. These oligonucleotides were analyzed by electrophoretic mobility shift assay. Purified p53 bound the labeled Bax oligonucleotide containing the entire 37-bp p53 response element of the *bax* promoter (Fig. 7*A*, *lane 1*), and this binding was effectively competed by an excess of the same, unlabeled oligonucleotide (Fig. 7*A*, *lanes 2–4*). Oligo BC, as well as Oligo AC failed to compete for p53 binding to any greater degree than Oligo B (Fig. 7*A*, compare *lanes 11–13* and *14–16* with *lanes 5–7*). Oligo AB, however, effectively competed for p53 binding (Fig. 7*A*, *lanes 8–10*). This competition was in the same range as that observed with the complete Bax oligonucleotide (Fig. 7*B*), suggesting that the two oligonucleotides share a similar affinity for the purified p53.

Each double-stranded oligonucleotide was inserted into the pGL3-E1bTATA reporter vector upstream of the adenovirus minimal *E1b* promoter and transfected into Saos-2 cells with either empty vector or the wild-type p53 expression vector (Fig. 8). Wild-type p53 effectively activated transcription through the 37-bp p53 response element of the *bax* promoter (60-fold) and to a lesser extent through Oligo B (13-fold). In contrast, p53 failed to significantly activate transcription through either Oligo A (2-fold) or Oligo C (1-fold). Consistent with the results of the electrophoretic mobility shift assays, p53 activated transcription through Oligo AB to a greater extent than through Oligo B (61-fold compared with 13-fold). This activation was in the same range as that observed with the complete p53 response element (61-fold compared with 60-fold). Both Oligos BC and AC failed to mediate any significant p53-dependent transactivation (4-fold and 1-fold respectively). These data confirm that in contrast to other p53 response elements, like the *p21*-5′ site, in which two adjacent p53 half-sites mediate transcriptional activation, the p53 response element of the *bax* promoter consists of three half-sites that cooperate to bring about full activation.

Two Nuclear Factors Selectively Interact with the p53 Response Element of the bax Promoter but Are Not Responsible for Its Differential Regulation in MDA-MB-453 Cells—Given that the defect in the ability of p53 to activate transcription of *bax* is at the level of the interaction between p53 and its response element in the *bax* promoter, one potential mechanism to explain the failure of p53 to activate transcription of *bax* in MDA-MB-453 cells might be that cellular factors exist in this cell line that can selectively compete p53 for binding to the *bax* promoter. To investigate this possibility, the labeled Bax oligonucleotide was used as a probe with MDA-MB-453 cell nuclear extract in an electrophoretic mobility shift assay. Four distinct nuclear factors bound this oligonucleotide (Fig. 8*A*, *lane 1*). Three of these factors, labeled *BoB1* and *BoB2* (binder of *bax* 1 and 2), and *n.s.*, were effectively competed by an excess of this same unlabeled oligonucleotide (Fig. 8*A*, *lanes 2–4*). The band labeled *n.s.* also was competed effectively by Oligos A, B, and C, as well as by the *p21*-5′ oligonucleotide (Fig. 8*A*, *lanes 2–16*), suggesting that this factor is a nonspecific (*n.s.*) DNA-binding protein. In contrast, the bands labeled *BoB1* and *BoB2* were effectively competed by an excess of unlabeled Oligo B but were not competed by Oligo A, Oligo C, or the *p21*-5′ oligonucleotide, demonstrating sequence specificity for Oligo B (Fig. 8*A*, compare *lanes 8–10* with *lanes 5–7* and *11–16*). The band shifts produced with nuclear extract of MDA-MB-453 cells were unaffected by the presence of anti-p53 antibodies (data not shown). In addition, BoB1 and BoB2 failed to bind the *p21*-5′ oligonucleotide, as well as oligonucleotides corresponding to the p53 response element of the *gadd45* gene and the 3′ element of the *mdm-2* gene (Fig. 8*A*, *lanes 14–16*, and data not shown). These results demonstrate the identification of two novel nuclear factors that display sequence specificity for the same region of the *bax* promoter that we have shown to be

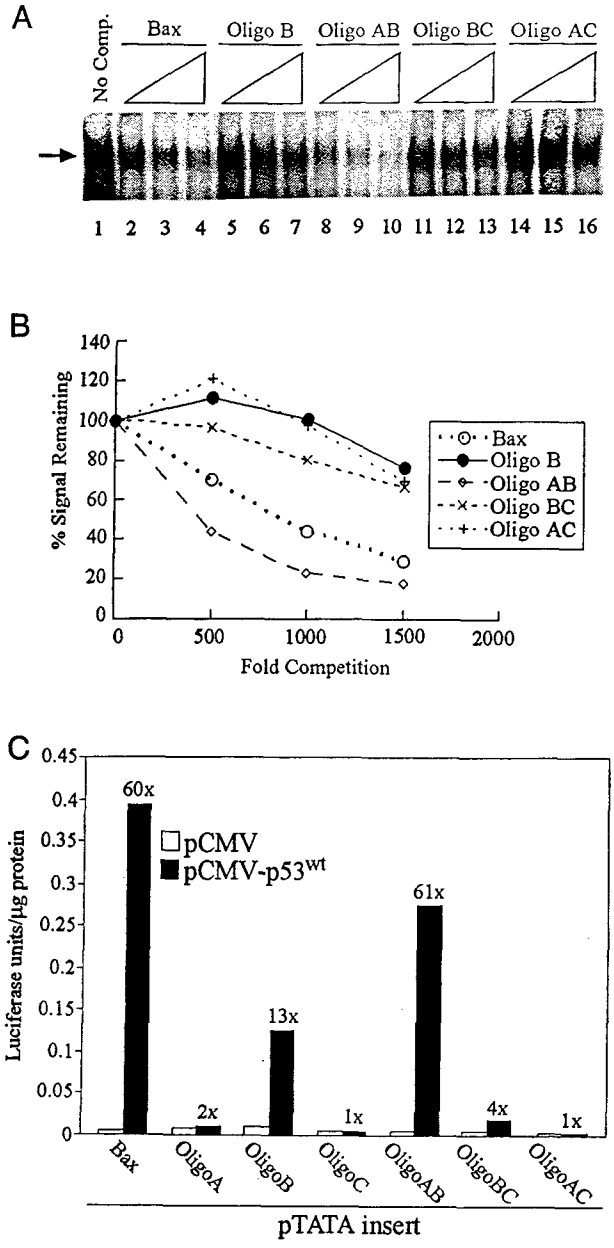

FIG. 7. **Overlapping binding sites synergize in p53 binding and in p53-dependent transactivation.** *A*, an electrophoretic mobility shift assay was performed using the Bax oligonucleotide as radiolabeled probe. 50 ng of purified p53 was incubated with 3 ng of the probe alone (*lane 1*) or in the presence of a 500- (lanes 2, 5, 8, 11, 14), 1000- (*lanes 3, 6, 9, 12,* and *15*), or 1500-fold (*lanes 4, 7, 10, 13,* and *16*) molar excess of the indicated unlabeled competitors. The *arrow* indicates the position of the p53-DNA complexes. *B*, bands were quantitated by densitometry and expressed as a percentage of the no competition signal (*lane 1*). *C*, Saos-2 cells were transfected as described under "Materials and Methods" with 2 μg of the indicated reporter constructs and 50 ng of either pCMV (*white bars*) or the wild-type p53 expression vector pCMV-p53^{wt} (*black bars*). 48 h post transfection luciferase activity and total protein levels were assayed as described under "Materials and Methods." The indicated values are the averages of three independent experiments each performed in duplicate. The *numbers* above each *black bar* indicate the fold activation for each pTATA construct observed with pCMV-p53^{wt} as compared with pCMV.

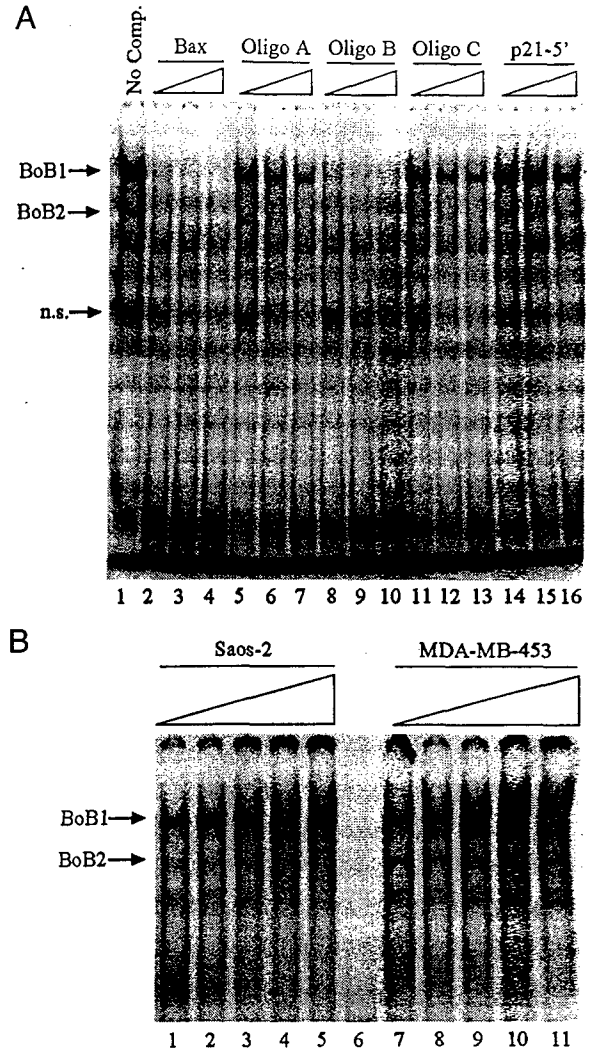

FIG. 8. **Nuclear extracts from MDA-MB-453 cells contain two factors that bind in a sequence-specific manner to the 37-bp p53 response element of the *bax* promoter.** *A*, an electrophoretic mobility shift assay was performed using the Bax oligonucleotide as radiolabeled probe. 2 μl (9 μg of total protein) of MDA-MB-453 nuclear extract was incubated with 3 ng of the probe alone (*lane 1*) or in the presence of a 10- (*lanes 2, 5, 8, 11,* and *14*), 50- (*lanes 3, 6, 9, 12,* and *15*), or 100-fold (*lanes 4, 7, 10, 13,* and *16*) molar excess of the indicated unlabeled competitors. *BoB1* and *BoB2* indicate the positions of the two sequence-specific DNA-binding factors, and *n.s.* indicates the position of a nonspecific band. BoB1 and BoB2 levels are equivalent in MDA-MB-453 and Saos-2 nuclear extracts. *B*, an electrophoretic mobility shift assay was performed using the Bax oligonucleotide as radiolabeled probe. 0 (*lane 6*), 4 (*lanes 1* and *7*), 8 (*lanes 2* and *8*), 12 (*lanes 3* and *9*), 16 (*lanes 4* and *10*), and 20 μg (*lanes 5* and *11*) of either Saos-2 (*lanes 1–5*) or MDA-MB-453 (*lanes 7–11*) nuclear extract was incubated with 3 ng of the probe. *BoB1* and *BoB2* indicate the positions of the two sequence-specific binding factors.

essential for p53-dependent transcriptional activation.

The identification of nuclear factors that showed sequence specificity for the p53 response element of the *bax* promoter suggests a potential mechanism for the differential activation of a reporter construct containing the *bax* promoter in MDA-MB-453 cells. To explore this possibility, the levels of BoB1 and BoB2 in Saos-2 (Fig. 8*B*, *lanes 1–5*) and MDA-MB-453 (Fig. 8*B*, *lanes 7–11*) nuclear extracts were compared by electrophoretic mobility shift assay, using the Bax oligonucleotide as radiolabeled probe. No significant difference in BoB1 or BoB2 levels was observed between nuclear extracts from these two cell lines (Fig. 8*B*, compare *lanes 1–5* with *lanes 7–11*) that had been normalized by total protein. These results suggest that BoB1 and BoB2 levels, as assessed by electrophoretic mobility shift assay, cannot explain the differential effects observed with wild-type p53 on its response element from the *bax* promoter in MDA-MB-453 cells as compared with Saos-2 cells.

The p53 Homolog p73 Does Not Selectively Inhibit the Ability of p53 to Activate Transcription through the bax Promoter—In

addition to BoB1 and BoB2, the p53 homolog p73 was examined as a potential explanation for the inability of wild-type p53 to activate transcription through the *bax* promoter in MDA-MB-453 cells. Saos-2 cells were transfected with a wild-type p53 expression vector, increasing amounts of an expression vector for p73α and either the p21P or pBax luciferase reporter constructs (Fig. 9). In the absence of p73, p53 activated transcription through both the *p21* (12-fold) and *bax* (48-fold) promoters. The addition of increasing amounts of p73 failed to inhibit the ability of p53 to activate transcription through either the *p21* or *bax* promoters, suggesting that p73 is not responsible for the differential activation observed with these two promoters in the MDA-MB-453 cell line.

An Intact C Terminus Is Not Required for p53-dependent Transcriptional Activation of the bax Promoter—Previous studies have demonstrated that C-terminal phosphorylation on serines 315 (47–49) and 392 (50) as well as acetylation of the C terminus (51) functionally alter the DNA binding characteristics of p53. Further, the ability of the C-terminal-specific mAb 421 to enhance the DNA binding activity of p53 has been proposed to be functionally similar to deletion of the last 30 amino acids of p53. In both cases, the binding of p53 to certain response elements is enhanced (50). As mAb 421 inhibits binding of p53 to the *bax* element, the effect of deletion of the terminal 30 amino acids was also examined. Saos-2 cells were transfected with either the p21P or pBax luciferase reporter plasmid and increasing amounts of pCMV-p53^{wt}, pB- p53^{S315A}, pB- p53^{S315D}, pCMV-p53^{S392A}, or pCMV-p53$^{\Delta370-393}$ expression vector (Fig. 10). In each case p53 effectively activated transcription through both the *p21* and the *bax* promoters, suggesting that neither phosphorylation of serine 315 or serine 392 nor an intact C terminus is required for the p53-dependent transactivation of the *bax* promoter. As compared with wild-type p53, each phosphorylation mutant activated transcription through the *p21* promoter to an equal or greater extent. Although these mutants, S315A, S315D, and S392A, also clearly activated transcription through the *bax* promoter (up to 18-, 16-, and 24-fold, respectively), this level of activation was consistently lower than that observed with the wild-type p53 (up to 72-fold), suggesting that although loss of phosphorylation on either of these residues alone does not completely inhibit the ability of p53 to activate transcription through the *bax* promoter they may contribute in a partial manner.

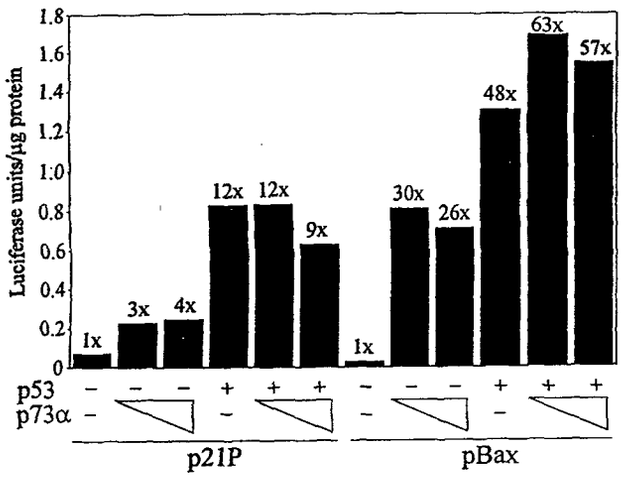

FIG. 9. **The p53 homolog p73 does not selectively inhibit the ability of p53 to activate transcription through the *bax* promoter.** Saos-2 cells were transfected as described under "Materials and Methods" with 2 μg of either the p21P or pBax luciferase reporter plasmids, 0 ng (–) or 50 ng (+) of pCMV-p53^{wt}, and 0 (–), 50, or 100 ng of pCMV-p73α. 48 h post transfection cells were lysed and assayed for total protein and luciferase activity as described under "Materials and Methods." Appropriate amounts of the vector pCMV were added to each transfection mixture to maintain a constant level of plasmid DNA of 2.1 μg/sample. The indicated values are the average of three independent experiments each performed in duplicate. The *numbers* above each *bar* indicate the fold activation for each reporter construct observed with pCMV-p53^{wt} and/or pCMV-p73α as compared with pCMV.

DISCUSSION

The data presented in this report demonstrate that wild-type p53 expressed in the osteosarcoma Saos-2 cell line successfully activated transcription through the promoters of both the cyclin-dependent kinase inhibitor *p21* and the proapoptotic *bax*. In contrast, p53 expressed in the breast carcinoma MDA-MB-453 cell line was capable of activating transcription through the *p21* promoter but failed to do so through the *bax* promoter (Fig. 1*A*). A luciferase reporter construct containing the 37-bp p53 response element from the *bax* promoter displayed the same differential response to p53 as the reporter containing the complete promoter (Fig. 2). This suggests that the 37-bp p53 response element alone is sufficient to mediate this differential regulation and argues in favor of the notion that the differential effect depends on an inherent difference in the interaction of p53 with its response elements in the *bax* and *p21* promoters. In this regard, the data demonstrate three distinct differences

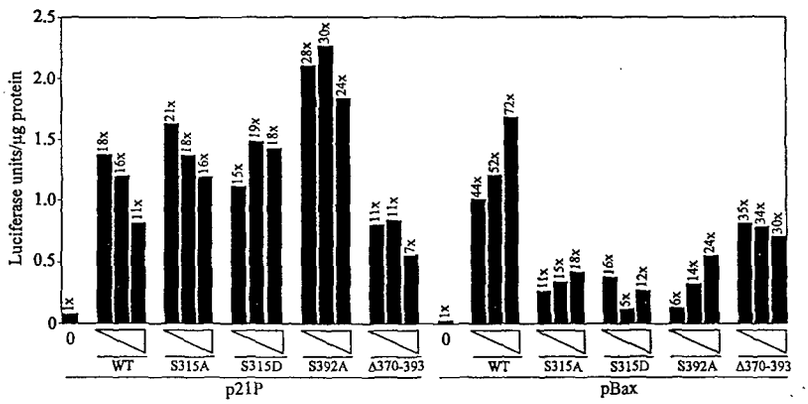

FIG. 10. **An intact C terminus is not required for p53-dependent transcriptional activation of the *bax* promoter.** Saos-2 cells were transfected as described under "Materials and Methods" with 2 μg of either the p21P or pBax luciferase reporter plasmids and 0, 50, 100, or 200 ng of pCMV-p53^{wt} (*WT*), pB-p53^{S315A} (*S315A*), pB- p53^{S315D} (*S315D*), pCMV-p53^{S392A} (*S392A*), or pCMV-p53$^{\Delta370-393}$ (*Δ370–393*). 48 h post transfection cells were lysed and assayed for total protein and luciferase activity as described under "Materials and Methods." Appropriate amounts of the vector pCMV were added to each transfection mixture to maintain a constant level of total plasmid DNA of 2.2 μg/sample. The indicated values are the average of three independent experiments each performed in duplicate. The *numbers* above each *bar* indicate the fold activation for each reporter construct observed with each p53 expression vector as compared with pCMV.

between the p53 response elements from these two promoters. First, unlike the *p21*-5′ element, which consists of two consensus p53 half-sites that form a high-affinity p53 response element, the response element of the *bax* promoter consists of three half-sites that cooperate in mediating p53-dependent transactivation (Fig. 7). Second, the studies with the C-terminal-specific mAb 421 suggest that the binding of p53 to its response element in the *bax* promoter, as compared with its binding to other response elements, involves a conformationally distinct form of p53 (Fig. 5). Finally, two novel nuclear factors, termed BoB1 and BoB2, were identified that demonstrated sequence-specific binding to the same region of the *bax* promoter that was essential for p53-dependent transactivation and failed to bind to the 5′ element of the *p21* promoter (Fig. 8).

The fact that the binding of p53 to the *bax* element, unlike that to the *p21*-5′ element, failed to be enhanced by the addition of mAb 421 (Fig. 5) indicates that the binding of p53 to these two sequences may require conformationally distinct forms of p53. Thus, the inability of p53 to activate transcription through the *bax* promoter in certain cell lines, like MDA-MB-453, may be due to an altered post-translational modification that prevents p53 from acquiring the correct conformation for binding. Alternatively, binding to the *bax* element may induce a distinct conformational change in p53, as compared with when it is bound to the *p21*-5′ element, that subsequently allows it to interact with a distinct set of additional regulatory factors, and the cell type-specific regulation is at the level of these additional regulators. This latter scenario has been observed with the transcription coactivator OCA-B. OCA-B is a B-cell-specific coactivator that markedly enhances transcription mediated by Oct-1 or Oct-2 through the octamer sequence of immunoglobulin promoters but fails to activate transcription mediated by the same Oct-1 or Oct-2 activators through octamer sequences in the histone H2B gene (52). Consistent with the notion that mAb 421 is revealing a conformational distinction significant to the observed differential regulation of *bax*, the ability of wild-type p53 to activate transcription through the *p21*-3′ response element, to which the binding of p53 also is inhibited by mAb 421 (46), was significantly decreased in MDA-MB-453 cells as compared with Saos-2 (Fig. 2).

Within the C terminus, phosphorylation of serines 315 (47–49) and 392 (50, 53–55) as well as acetylation of lysines 370, 372, and 373 (51) have been shown to enhance the DNA binding (47–51), transcriptional activation (53, 54), and growth suppressor (55) functions of p53. In fact, Scheidtmann and coworkers (49, 54) have suggested that phosphorylation of serines 315 and 392 alters the ability of p53 to both bind to and activate transcription through the p53 response element of the *bax* promoter, in particular. Given these results and the observation that the C-terminal-specific mAb 421 inhibits the binding of p53 to the *bax* element (Fig. 5), we investigated whether or not these particular post-translational modifications could explain the observed defect in the ability of wild-type p53 to activate transcription through the *bax* promoter in the MDA-MB-453 cell line. The results in Fig. 10 demonstrate that although mutation of either serine 315 or serine 392 to alanine slightly decreases the ability of p53 to activate transcription through the *bax* promoter, as compared with the *p21* promoter, Thus, neither phosphorylation of 315 or 392 nor an intact C terminus is required for p53 to effectively activate transcription through either the *bax* or *p21* promoters. Because the data presented here address each modification independently of the others, the possibility still exists that some combination of these modifications, or other C-terminal modifications not addressed here, may have a more significant impact on the ability of p53 to activate transcription through the *bax* promoter.

The identification of two novel nuclear factors, BoB1 and BoB2, that showed sequence specificity for the same region of the *bax* promoter that was essential for p53-dependent transactivation (Figs. 6 and 8) suggested an alternate explanation for the observed defect in MDA-MB-453 cells. Preliminary results indicated that the binding of p53 and BoB1 or BoB2 to the p53 response element of the *bax* promoter were mutually exclusive, suggesting that these factors may compete with p53 for binding (data not shown). These factors demonstrated a strong affinity for the *bax* element and poor affinity for the *p21*-5′ element. In addition, BoB1 and BoB2 were found to display a moderate affinity for the *p21*-3′ element (data not shown). Correspondingly, the level of p53-dependent activation of the reporter construct containing this 3′ element was reduced in MDA-MB-453 cells when compared with its level of activation in Saos-2 cells (Fig. 2). These results suggested an inverse relationship between the affinity of these binding factors for a particular sequence and the ability of that sequence to mediate p53-dependent transcriptional activation in MDA-MB-453. When the levels of these factors in MDA-MB-453 and Saos-2 cells were compared, however, there was no discernable difference observed (Fig. 8*B*), suggesting that although these factors still may have some significance to the p53-dependent transactivation of *bax*, they do not explain the observed defect in the MDA-MB-453 cell line. One could hypothesize that the p53 homolog p73 might function in a manner analogous to that originally proposed for the BoB1 and BoB2 binding factors. Given the sequence homology between the DNA-binding domains of p53 and p73, it is reasonable to speculate that p73 can bind DNA at p53 response elements and, therefore, may compete with p53 for binding. The results presented here, however, do not support such a hypothesis. Expression of p73α was unable to inhibit the ability of p53 to activate transcription through either the *bax* or *p21* promoters (Fig. 9). In fact, p73 was found to be a potent activator of transcription through the *bax* promoter (Fig. 9, up to 30-fold).

The identification of tumor-derived p53 mutants that selectively fail to activate transcription through the *bax* promoter and subsequently fail to undergo apoptosis (36–39) suggests that the ability of p53 to activate transcription through the *bax* promoter is important to the tumor suppressor function of p53. The Bax protein, in fact, has been shown to play an important role both in inhibiting tumor progression and in promoting the apoptosis of tumor cells in response to DNA-damaging agents like those used in the treatment of cancer (56–62). Studies have shown that decreased Bax levels are significantly associated with tumor cell resistance to chemotherapy (56, 58) and that increased expression of Bax is sufficient to sensitize at least certain tumor cell types to apoptotic stimuli (57, 60, 61, 63). In addition, the p53-dependent transcriptional activation of the *bax* gene has been shown to be important both in inhibiting tumor formation and progression (59, 62, 64) and in promoting apoptosis in response to radio and chemotherapy (59, 63). As such, understanding the mechanism of p53-dependent regulation of the *bax* gene will provide new insights into the processes of tumor formation and progression, as well as the development of tumor resistance to treatment. The data presented here identify several characteristics that differentiate the p53 response element of the *bax* promoter from other p53 response elements, such as the *p21*-5′ element. These characteristics suggest a potential mechanism for the cell type-specific regulation of the *bax* promoter by p53, as seen with the MDA-MB-453 and Saos-2 cell lines. The data demonstrate that in this model system the defect in the ability of wild-type p53 to activate transcription through the *bax* promoter is at the level of the interaction between p53 and its response element and

that this interaction appears to involve a conformationally distinct form of p53 interacting with a unique arrangement of three half-sites. It is reasonable to speculate that the mechanism responsible for the failure of wild-type p53 to activate transcription through the *bax* promoter in MDA-MB-453 cells may also be relevant to the inhibition of *bax* induction observed both in tumor formation and progression and in tumors that are resistant to apoptosis-inducing treatments.

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TARGET GENE SELECTION BY p53 IS REGULATED BY MULTIPLE MECHANISMS

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Depending upon particular cellular conditions, the tumor suppressor protein p53 induces growth arrest or apoptosis. Since the DNA binding activity of p53 plays a role in each of these responses, the ability of p53 to select among various target genes to elicit a particular cellular outcome may be central to the regulation of its biological function. Three mechanisms have been identified which can contribute to the regulation of target gene selection by p53.

First, two classes of p53 response elements have been identified by examining the effect of monoclonal antibody 421 (mAb 421) on the sequence-specific DNA binding of p53 in electrophoretic mobility shift assays (EMSA). Incubation with mAb 421 enhanced the binding of p53 to one set of response elements but inhibited binding to another set. A comparison of these elements as well as mutational analysis of sites from the *p*21 promoter has defined some of the sequence determinants that distinguish the two classes of elements. Further, this ability of mAb421 to either enhance or inhibit DNA binding by p53 was dependent on the presence of non-specific high molecular weight DNA and could be regulated by particular high mobility group proteins. The results are consistent with p53 adopting distinct conformations when bound to different subsets of response elements.

Second, two novel factors which bind to a subset of p53 response elements in a sequence-specific manner have been identified. These factors appear to be distinct from p53 and its homologs. EMSA studies have suggested that the binding of p53 and these nuclear factors may be mutually exclusive, suggesting that the interaction of these factors with a subset of p53 response elements is a mechanism for regulation of p53 target gene selectivity.

Third, additional sequence elements besides the p53 binding sites appear to be involved in p53-dependent regulation of the *p*21 and *cdc*25C genes. The 3' site in the *p*21 promoter requires an additional element to confer p53-dependent activation on a minimal promoter. The p53 site in the *cdc*25C promoter confers p53-dependent activation, however, the presence of an additional *cdc*25C promoter element causes p53 to repress transcription through the same p53 binding site. Thus, sequence context can determine the functional consequences of p53 binding.

Understanding the basis for target gene selection by p53 has implications for cancer treatment. The optimal therapeutic response to DNA damage caused by chemotherapeutic agents is apoptosis rather than cell cycle arrest. Elucidating the molecular mechanisms that regulate target gene selectivity by p53 may give insight into the ability of p53 to trigger apoptosis versus arrest and lead to more effective therapeutic intervention.